SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

GENETIC ALTERATIONS IN CRANIOSYNOSTOSIS, GENOTYPE-PHENOTYPE CORRELATIONS

by

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The Examination takes place at the Department of Ophthalmology, Faculty of Medicine, University of Debrecen, 10 December, 2015., 12:00 p.m.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 10. december 2015., 13:30.

1. INTRODUCTION

Craniosynostoses, caused by the premature fusion of cranial sutures, are characterized by abnormal skull shape. They occur in two main forms: in the isolated (non-syndromic) type, craniosynostosis is not associated with other clinical signs, while in syndromic cases, facial dysmorphism and limb anomalies can be observed as well.

Deformity of the skull is not only an aesthetic problem, but - depending on the type and etiology of the disease -, it may lead to serious neurological, ophthalmological or respiratory consequences requiring early surgical interventions. Therefore, early recognition and treatment of craniosynostosis as well as determination of the subtypes are of great clinical significance. Besides physical examination and applying imaging techniques (roentgen, CT), identification of the genetic background is also important in the diagnostics of craniosynostoses, in separation of isolated and syndromic forms and classification of patients. Genetic alterations detected have a prognostic value and allow prenatal genetic testing in further pregnancies; on the basis of the result, parents can decide upon the outcome of the pregnancy.

Our knowledge of the clinical and genetic characteristics of patients with craniosynostosis in Hungary is incomplete. Identification of the syndromic forms is a diagnostic challenge because of their rarity and variable expressivity. A clinical center with experts experienced in diagnostics and treatment of craniosynostoses, formed in 2005 in Hungary, can provide a good opportunity for targeted and centralized medical care of these patients. From that time, craniofacial reconstructions have been performed in the Department of Neurosurgery, University of Debrecen, while the Department of Pediatrics provides the pre- and postoperative treatment and genetic diagnostics of patients with the contribution of neonatologists, pediatricians and clinical geneticists. This situation provides a great

opportunity to overview and summarize our experience in the clinical and genetic characteristics of craniosynostosis and genotype-phenotype correlations.

2. LITERATURE REVIEW

Craniosynostoses, the premature fusion of cranial sutures, are characterized by the deformity of the skull. It is one of the most frequent form of craniofacial disorders with an incidence of 1:2100-2500 births. Early fusion of the sutures usually begins prenatally, thus craniosynostosis can be diagnosed mostly in newborns. In the isolated (non-syndromic) forms (80-85% of patients), besides the cranial deformity and its potential consequences (e. g., increased intracranial pressure, brain blood flow, respiratory distress, visual and hearing impairment), no other specific clinical signs could be observed. In the contrary, in syndromic forms the cranial abnormality often associates with further specific symptoms including various facial dysmorhic signs and limb anomalies, however, there is an overlapping between the phenotypes. More than 100 syndromes associated with craniosynostosis are known nowadays.

2.1 Etiology of craniosynostosis

The isolated forms are mostly sporadic, their etiology is not entirely clarified yet. They are considered to be of multifactorial origin: both the genetic background and environmental factors may play an important role in their development. By epidemiological studies, the main risk factors of isolated craniosynostoses are - among others- the male sex, high (>4000g) or low (<1500g) birth weight, preterm (<37 week) birth, breech presentation, plurality and advanced (>35 years) maternal age. Smoking, medication (e. g. antiepileptic drugs) or alcohol use during pregnancy may also predispose to craniosynostosis. In contrast

with the isolated group, syndromic forms are mostly caused by genetic alterations, which can be gene mutations or chromosomal aberrations.

2.1.1. Gene alterations

In about 25% of craniosynostoses, pathogenic mutations can be detected in genes playing important role in the differentiation and proliferation of osteoblasts and ossification. The most frequently involved genes in craniosynostosis are those encoding the fibroblast growth factor receptors and the TWIST1 (twist family basic helix-loop-helix transcription factor 1) transcription factor.

Fibroblast growth factor receptors (FGFR) and their genes

The four members of FGF receptor family (FGFR1-4) are transmembrane signal transduction molecules with tyrosine kinase activity. They consist of an extracellular ligand binding part with 3 immunoglobulin-like domains (IgI, IgII, IgIII), a transmembrane domain and a splitted intracellular tyrosine kinase domain. The IgIII domain is located in the center of the ligand binding part of the receptor, thus its amino acid sequence is crucial for the specificity of ligand binding. The ligands of the receptors are the FGF molecules consisting of 23 members, the ligand specificity of the receptors is different. They play an important role in organogenesis, neurogenesis, wound heeling and endochondral or intramembranous ossification.

In the development of craniosynostosis, *FGFR1-3* genes are involved. Heterozygous, activating mutations of *FGFR2* can be identified in several syndromes such as Apert, Crouzon or Pfeiffer syndromes. Most of these mutations are missense and located in the two hotspot regions (exon IIIa and IIIc) of the gene. The p.Pro252Arg mutation in *FGFR1* is specific for Pfeiffer syndrome type 1, while in the *FGFR3* gene, 2 mutations are known to

cause syndromic craniosynostosis (p.Pro250Arg, Muenke syndrome; p.Ala391Glu, Crouzon syndrome with acanthosis nigricans). FGFR mutations cause gain of function, the receptors become activated constantly. Enhanced signal transduction results in increased proliferation and differentiation inducing intense ossification.

TWIST1 transcription factor and its gene

The TWIST1 molecule, a member of the basic helix-loop-helix transcription factor family, has a crucial role in the development of the skeleton as a key molecule of mesenchymal cell death. It has a positive or negative effect on the proliferation, differentiation and survival of osteoblasts through the regulation of several signal transduction pathways. It regulates the development of the cranial sutures indirectly through the BMP (bone morphogenetic protein) and FGF pathways. Heterozygous, loss-of-function mutations in the coding exon of *TWIST1* are various: they can be missense, nonsense or frameshift mutations. In certain cases, deletion of the whole gene or larger region can be found leading to microdeletion. *TWIST1* mutations cause craniosynostosis by haploinsufficiency.

The common craniosynostosis syndromes show autosomal dominant inheritance. In syndromes with serious phenotype (e. g., Apert or Pfeiffer syndrome type 2), new mutations are expected, while in milder syndromes (e.g., Muenke or Crouzon syndromes) familial cases can also be observed.

2.1.2 Chromosomal abnormalities

Several chromosomal aberrations have been described in craniosynostosis, most of them are sporadic. One part of the cytogenetic aberrations involves the 7p21.1 region containing the *TWIST1* gene, which translocation or deletion cause Saethre-Chotzen syndrome. Other

aberrations include terminal 9p, 11q23 or 22q11 deletions, 1p36 or 5q35 trisomies, etc. In certain cases without chromosomal aberrations, various submicroscopic alterations may be detected with multiplex ligation dependant probe amplification or array comparative genome hybridization (arrayCGH). Chromosomal aberrations constitute about 15% of genetically proved cases.

3. AIMS OF THE STUDY

1. The aim of our study was to investigate the clinical and genetic aspects of patients with syndromic craniosynostosis referred to the Department of Pediatrics and Department of Neurosurgery, and establish genotype-phenotype correlations.
2. We intended to use cytogenetic, molecular cytogenetic and molecular genetic methods to detect genetic aberrations.
3. In the case of a new mutation, additional analyses were planned to use to confirm its pathogenecity.
4. In the knowledge of the genetic aberration and the clinical signs, we studied whether there is a correlation between the genotype and the phenotype, the severity and the outcome of the surgical intervention.
5. In familial cases, examination of the proband was complemented with the investigation of family members to enhance the better recognition of the rarer syndromes.
6. On the basis of our results, we aimed to establish a genetic algorithm, which can be effectively used in diagnostics.

7. Besides the investigation of the syndromic forms, we studied the frequency of different types of isolated craniosynostosis, and the role of selected perinatal factors in the development of the disorder.

4. PATIENTS AND METHODS

4.1.Patients

Two hundred patients with craniosynostosis were referred to the Department of Neurosurgery and Department of Pediatrics, University of Debrecen between the years 2006 and 2012. Synostosis of the sutures was confirmed by either X-ray or computed tomography, and the syndromic nature of the disease was established by a team of pediatricians and clinical geneticists. Of the 200 enrolled patients, 198 were under 10 years of age with a median age of 6 months (1 month-10 years), while only 2 patients were adults (18 and 28 years old). Reconstructive operative procedures were performed in 195 patients. In 24 patients, clinical signs other than the malformed skull suggested the syndromic forms of the disease. Patients diagnosed with craniosynostosis syndromes during the seven years of the study had the following types: Apert (n=5), Pfeiffer (n=5), Muenke (n=4), Crouzon (n=2) and Saethre-Chotzen (n=1) syndromes. Multiple-suture craniosynostosis associated with achondroplasia was found in one patient (n=1). Phenotypic features were not typical for any particular syndrome in six patients.

4.2. Methods

To investigate the etiology of the disease genetic analyses were performed only in patients with clinically identified or suspected syndromic craniosynostosis and their relatives showing the clinical signs of a specific syndrome. Genetic testing included the molecular

analysis of the mutational hotspots of the *FGFR1*, *FGFR2*, *FGFR3* and *TWIST1* genes, G-banded karyotyping and fluorescence in situ hybridization (FISH) analysis of *TWIST1*. If a specific syndrome was identified (n=18), targeted analysis was performed, while in suspected syndromic cases (n=6) all of the previously mentioned tests were applied. In one patient arrayCGH analysis was also performed. After obtaining written informed consent, blood samples were taken from 24 syndromic patients and 8 relatives. To assess the pathogenecity of the novel mutation, DNA samples of 50 healthy, controll persons were analyzed.

4.2.1. DNA extraction

To examine the mutational hotspots, genomic DNA was extracted from the patients' peripheral blood using the QIAamp DNA Blood Mini kit (Qiagen, Germantown, MD) according to the manufacturer's instruction.

4.2.2. Mutational analysis of the FGFR1, 2, 3 and TWIST1 genes

Amplification of exon 7 of *FGFR1*, exons 8 and 10 of *FGFR2*, exons 7 and 10 of *FGFR3*, and the coding region of exon 1 in *TWIST1* was performed by polymerase chain reaction (PCR) with previously published primer pairs. For DNA sequencing, the PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Germantown, MD, USA). Purified PCR products were sequenced on the ABI 3100 sequencer (Applied Biosystems, Foster City, CA, USA) with the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). Electropherograms of the sequenced products were compared to reference sequences.

4.2.3. Assessing the pathogenicity of the novel variant

To assess the pathogenicity of the novel variant, c.528C>G (p.Ser176Arg) in exon 1 of *TWIST1*, SIFT (http://sift.jcvi.org) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) predictions were performed. Alignment analysis of TWIST1 proteins was carried out using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The presence of the novel variant was tested by restriction fragment length polymorphism (RFLP) analysis using BspMI (Thermo Scientific, Waltham, MA, USA) restriction enzyme in 50 healthy individuals.

4.2.4. Cytogenetic analysis

To detect the constitutional karyotype of the patients, conventional karyotyping was performed. Blood samples (0.5 mL) of patients anticoagulated with Na-heparin were cultured in medium (5 mL, Lymphochrome Medium, Lonza, Belgium) during 72 hours, at 37 degrees in a CO_2 (5%) incubator. To interrupt cell division, colchicin (0.5 ug/ml, Sigma-Aldrich, St. Louis, MO, USA) was added to the samples, and after incubation (1 hour), hypotonization of the samples were performed with 0,075 M KCl solution. Fixation of the samples was performed with methanol-acetic acid (3:1) solution. For chromosome banding, treatment with trypsin (Sigma-Aldrich, St. Louis, MO, USA) was used followed by Giemsa staining (Merck, Darmstadt, Germany). We evaluated 10 metaphases with Lucia Karyo software (Lucia Cytogenetics, Czech Republik) in every patient. Determination of the karyotype was based on the International System for Human Cytogenetic Nomenclature (ISCN 2005 and 2009).

4.2.5. FISH analysis of TWIST1

FISH analysis was performed using cell suspension derived from chromosome preparation. After dropping and aging (37 ºC, one hour), slides were pretreated with the following solutions: 2xSSC/0.5% NP40, 37 ºC, 15 minutes; pepsin treatment, 37 ºC, 5 minutes; 1x PBS, room temperature, 5 minutes. After dehydration (70%-85%-100% ethanol, 2-2 minutes) and drying, 1.5 µl probe (Williams-Beuren/Saethre-Chotzen probe, Cytocell, Cambridge, UK) was dropped onto the slide, and it was covered by a coverslip. Codenaturation of the sample and the probe was performed at 76 ºC for 3 minutes, and hybridization was performed at 37 ºC, overnight in a hybridization machine (Hybrite, Abbott/Vysis, Des Plaines, IL, USA). We washed the unbounded probe in 50% formamid/2X SSC solution at 42 °C, for 15 minutes, in 2X SSC solution at room temperature for 10 minutes and in 2X SSC/ 0,1 % NP40 solution at room temperature for 5 minutes. After drying, cell nuclei were stained with 4'-6' diamino-2-fenil-indol (DAPI, Abbott/Vysis, Des Plaines, IL, USA). Evaluation of the samples was performed with Zeiss Axioplan2 microscope (Carl Zeiss, Jena, Germany) and ISIS software (Metasystems, Altlussheim, Germany). At least 15 metaphases were evaluated in every patient.

4.2.6. ArrayCGH analysis

ArrayCGH analysis was performed in a patient with multiple-suture craniosynostosis and achondroplasia at the Department of Clinical Genetics, Academic Medical Centrum, Amsterdam, Netherlands with Agilent 180K oligo-array, Amadid 023363 (Agilent Technologies, Inc., Santa Clara, CA, USA). Standard methods were used for labelling and hybridization, array profiles were evaluated by Agilent software.

4.2.7. Statistical analysis

To assess the potential risk factors for craniosynostosis, selected perinatal data (sex, plurality, gestational age at birth and birth weight) of 142 non-syndromic patients were compared with the general Hungarian live births data (average data of 2005-2010 years) provided by the Hungarian Central Statistical Office. Statistical analyses included Chi-square and Fisher's exact tests performed by IBM SPSS 20 (IBM Corporation, Armonk, New York). The results were considered to be significant at a < 0.05 significance level.

5. RESULTS

Two hundred patients with craniosynostosis were enrolled in the study. On the basis of detailed clinical assessment, the condition proved to be isolated (nonsyndromic) in 176 of 200 (88%) patients while 24 cases (12%) were syndromic.

5.1. Clinical symptoms of syndromic patients

Clinical signs in patients with *Apert syndrome* included brachycephaly due to bicoronal synostosis, high and broad forehead, midface hypoplasia, depressed nasal bridge, convex nasal ridge, hypertelorism and syndactyly of fingers and toes. In the majority of patients, syndactyly of all toes on both feet and syndactyly of the 2/3/4/5 fingers on both hands were observed (Patients 1-4). In Patient 5, complete syndactyly of all fingers and all toes could be seen. Cleft palate was observed in one patient.

Both patients with *Crouzon syndrome* showed brachycephaly, proptosis, flat nasal bridges, mandibular prognathism and low-set ears. In one of them acanthosis nigricans was also present.

Three of the patients with *Pfeiffer syndrome* had severe cranial malformation (cloverleaf or "Kleeblattschädel" skull), hydrocephalus, extreme proptosis, low-set ears and short, small noses. Broad great toes were observed in all of these patients, while the thumbs were broad only in two of them. Limited extension of the elbows was also noticed in two patients. In a patient, medially deviated great toes, the characteristic feature of Pfeiffer syndrome, were observed and were associated with partial 2/3 syndactyly on the feet. Based on the phenotypic signs, the latter three patients were considered to have Pfeiffer syndrome type 2. In two further patients, Pfeiffer syndrome type 1 was diagnosed based on craniofacial anomalies milder than those in patients with type 2. One of them had brachycephaly, a high and broad forehead, exophthalmos, broad thumbs, 3/4 syndactyly of the fingers, broad great toes and 2/3/4 syndactyly on the toes. The other patient had brachy- and acrocephaly, high forehead, wide and depressed nasal bridge, hypertelorism, long philtrum, thin lips, low set ears with overfolded helices. Limb anomalies included broad thumbs, broad great toes with varus deformity, complete 3/4 cutaneous syndactyly of the right foot and 2/3/4 partial cutaneous syndactyly of the left. Detailed clinical and genetic investigations of the other patient and her 4 family members were performed.

All of the *Muenke syndrome* patients had brachycephaly resulting from the synostosis of the coronal sutures, high and flat foreheads, hypertelorism and almond-shaped eyes. High-arched palate was seen in two of them. Limb abnormalities were variable, including broad thumbs and great toes, clinodactyly of the fifth fingers and capitate-hamate fusion. The mothers of three patients showed similar phenotypes to those of their children, but their clinical signs seemed to be milder.

Typical clinical signs of *Saethre-Chotzen syndrome* were observed in a patient, who had brachycephaly, an asymmetric flat face, long and deviated nose, thin lips, ptosis of the right

eyelid, shallow orbits, and broad and bifid great toes. The mother of the patient also had brachycephaly, a long nose, thin lips and bifid great toes; additionally, her thumbs were broad.

Multiple-suture craniosynostosis associated with rhizomelic shortening of the limbs and trident hands, the typical clinical signs of achondroplasia were found in a patient.

In six patients, additional clinical features associated with craniosynostosis included various facial dysmorphic signs and limb alterations suggesting the syndromic form of craniosynostosis; however, the phenotypes of these patients were not typical for any particular syndrome.

5.2. Genetic alterations in syndromic patients

The *Apert and Pfeiffer syndromes* could most commonly be observed in the syndromic group. Genetic abnormalities were detected in 75% (18/24) of the syndromic patients and in 8 relatives showing the clinical signs of a specific syndrome. All mutations were heterozygouos.

In 4 patients with *Apert syndrome*, we identified the c.758C>G (p.Pro253Arg) mutation in *FGFR2*, while in one patient the c.755C>G (p.Ser252Trp) mutation of the same gene could be detected. Both mutations are specific for this syndrome.

In 4 patients with *Pfeiffer syndrome*, the *FGFR2* gene was involved: in three cases various changes of the 342. amino acid were detected (c.1024T>C, p.Cys342Arg és c.1025G>C, p.Cys342Ser), while in one patient a splicing mutation (c.940-1G>A) could be identified. The rare c.755C>G p.Pro252Arg mutation of *FGFR1* was detected in the patient with Pfeiffer syndrome type 1 characterized by mild symptoms. Detailed clinical genetic

examination of the family was performed, and the mutation was proved in further 4 family members.

In one of the patients with *Crouzon syndrome* the c.833G>T (p.Cys278Phe) mutation of *FGFR2* was detected, in the other patient with acanthosis nigricans, the c.1172C>A (p.Ala391Glu) mutation in the *FGFR3* gene was found.

The mutation specific for *Muenke syndrome*, the c.749C>G (p.Pro250Arg) in *FGFR3*, could be detected in 4 patients, three of them inherited it from their mothers.

In the patient with achondroplasia and multiple-suture craniosynostosis, the achondroplasia-specific c.1138G>A (p.Gly380Arg) mutation was found. Any other genetic alteration which may have been related to the associating craniosynostosis, could not be identified by sequencing of the hotspot regions of *FGFR1-3*, G-bandig karyotyping, FISH and arrayCGH.

In addition to 10 different, known mutations detected in *FGFR1-3*, one previously undescribed missense mutation, the c.528C>G (p.Ser176Arg), was found in the *TWIST1* gene of a patient with Saethre-Chotzen syndrome.

5.3. Assessing the pathogenecity of the new mutation p. Ser176Arg

The pathogenicity of the new mutation, c.528C>G (p.Ser176Arg), is supported by the following arguments:

1. the affected residue is phylogenetically highly conserved in human, mouse, zebrafish and xenopus;
2. according to SIFT and PolyPhen-2 predictions, this missense alteration was predicted to be damaging and probably damaging, respectively;
3. RFLP analysis of exon 1 of *TWIST1* showed that this mutation was not present in 50 healthy individuals representing 100 alleles.

4. The mother, who had very similar phenotype also had the novel c.528C>G (p.Ser176Arg) mutation.

5.4. The role of selected perinatal risk factors in non-syndromic craniosynostoses

In the isolated group, the most frequently fused suture was the sagittal one (120/176; 68%) followed by the coronal (26/176; 15%), metopic (18/176; 10%) and lambdoid sutures (7/176; 4%) in this order. In 5 patients (3%), more than one suture was involved. The male-to-female ratio was 2.2:1, showing a male predominance.

Perinatal data of 142 nonsyndromic patients were compared to the general Hungarian live birth data to assess their possible associations with craniosynostosis. Male sex ($P< 0.001$), twin gestation ($P< 0.001$) and very low (< 1500 g) birth weight ($P< 0.001$) proved to be risk factors for nonsyndromic craniosynostosis. Being male ($P< 0.001$) and twin gestation ($P=0.001$) seemed to be associated with sagittal synostosis, while very low birth weight was a predictor for coronal ($P<0.001$) synostosis. No association between metopic synostosis and any risk factor analyzed in the study could be confirmed. Because of the small number of patients, no statistical analysis could be performed on lambdoid synostosis.

6. DISCUSSION

Our study is the first nation-wide investigation providing clinical and genetic information on craniosynostoses. Similarly to previously published studies, nonsyndromic patients represented the majority of all cases, while syndromic forms constituted only the 12% of craniosynostoses.

6.1. Genetic alterations in syndromic craniosynostoses

Genetic abnormalities were detected in syndromic patients with clinical signs characteristic for a specific syndrome.

6.1.1. Genotype-phenotype correlations

Apert syndrome is one of the most serious craniosynostosis syndromes with specific clinical signs. According to the literature, the specific mutations p.Ser252Trp and p.Pro253Arg of *FGFR2* can be detected in about 99% of patients with Apert syndrome, while Alu-element insertions in *FGFR2* are a rare cause of this condition. In our study, all patients with Apert syndrome carried one of the two specific mutations. While the p.Ser252Trp mutation could be detected in only one patient in our study, other authors found this mutation with a higher frequency. Regarding the clinical features, cleft palate has been reported to be more common with the p.Ser252Trp mutation, while syndactyly was found to be more severe in patients with the p.Pro253Arg mutation. Our result supports this observation: the only patient, who had cleft palate, carried the p.Ser252Trp mutation.

Pfeiffer syndrome is known to be clinically and genetically heterogenous disorder. The two ends of the phenotypical spectrum are represented by the very mild, almost unrecognizable cranial deformity on one hand and the severe cloverleaf skull on the other. The mutations eliminating the cysteine 342 residue in FGFR2 have a different phenotypic impact depending on the type of amino acid exchange. The conversion of cysteine to phenylalanine or tyrosine mainly results in Crouzon syndrome, whereas arginine substitution preferentially causes Pfeiffer syndrome with severe cranial manifestation and poor prognosis. In our cohort, two patients with Pfeiffer syndrome harbored the p.Cys342Arg mutation; both of them had cloverleaf skulls requiring two and three reconstructive surgical interventions,

hydrocephalus, severe exophthalmos, and respiratory and auditory problems. In addition, exchange of the same amino acid residue for serine caused very similar, severe phenotype in the third patient with Pfeiffer syndrome type 2. The mild form of Pfeiffer syndrome, the type 1 was observed in two patients. In one of them, splicing mutation of *FGFR2* (c.940-1G>A) was identified, while the other patient had the p.Pro252Arg mutation in *FGFR1*.

In 1994 Muenke et al. identified the *FGFR1* gene on chromosomal region 8p11 to be associated with Pfeiffer syndrome. A specific mutation, p.Pro252Arg, located in the IgII-III linker region of FGFR1, has been reported to cause mild symptoms making the diagnosis rather difficult in many cases. *FGFR1* p.Pro252Arg mutation without craniosynostosis has been reported on two occasions in the literature providing a further evidence of variable expressivity of the syndome. Phenotypic features in the family with the mutation above showed high variability ranging from apparently normal skull and limbs to characteristic brachycephaly and digital anomalies. Typical features of the syndrome appeared only in the third generation, suggesting that this condition is underdiagnosed in many cases. In the case of *FGFR1* p.Pro252Arg mutation, a variable expressivity can b e expected which in mild cases makes the diagnosis difficult. The case of this family emphasizes the significance of detailed physical examination of not only the proband but the family members as well, because the specific symptoms of the syndrome may occasionally appear only after several generations.

Crouzon syndrome is usually caused by *FGFR2* mutations. Association of the syndrome with acanthosis nigricans is very rare and considered to be a separate entity called *Crouzonodermoskeletal syndrome* because of its specific phenotype and genotype. The specific alteration of the syndrome is the c.1172C>A (p.Ala391Glu) mutation of *FGFR3*. In contrast to the common *FGFR1* and *2* mutations located in the extracellular part of the

receptor, this mutation is found in the transmembrane region of FGFR3 enhancing signalization by the stabilization of receptor dimers. In one patient with Crouzon syndrome, a common mutation of the syndrome, c.833G>T, p.Cys278Phe in *FGFR2,* was found, while in the other patient acanthosis nigricans was also present and the former specific mutation was detected. Because of the former observations, besides cranial deformity and facial dysmorphism we must pay special attention to the skin, but limb anomalies are not expected. *Muenke syndrome* is characterized by the synostosis of the coronal suture, very mild facial dysmorphism, limb anomalies which can be seen sometimes only by X-ray imaging and the presence of the specific *FGFR3* p.Pro250Arg mutation. The syndrome was identified in 4 patients, three probands inherited the mutation from their mothers. Recent international studies recommend the mutational analysis in every patient with isolated craniosynostosis involving the coronal suture in order to detect the very mild, almost unrecognizable assocating synostosis and facial dysmorphy. In a patient with *Saethre-Chotzen syndrome* we succeeded to identify a novel missense mutation, p.Ser176Arg, in *TWIST1*. TWIST1 is a basic helix-loop-helix transcription factor involved in a variety of signal transduction pathways in tissues of mesodermal origin. It has a crucial role in the migration and differentiation of cranial neural crest cells during cranial development. The novel alteration is located between the highly conserved bHLH and Twist box domains. Although this linker region is evolutionarily conserved across a wide range of vertebrate species, its functional importance is still unclear. Pathogenecity of the variant is supported by the conservity of the 176. amino acid of the human TWIST1, the probable damaging effect of serine-arginine exchange, the lack of the mutation in the healthy control population examined, and its presence in the mother showing the clinical signs of the syndrome.

6.1.2. Association of craniosynostosis with achondroplasia

Association of achondroplasia, as the most common form of chondrodysplasias, with craniosynostosis is extremely rare, only 3 cases have been reported before our publication. In our patient, acrocephaly and left posterior plagiocephaly was observed due to multiple-suture craniosynostosis. The achondroplasia-specific mutation could be detected, but a second pathogenic mutation which may have lead to the simultaneously existing craniosynostosis could not be identified in the hot-spot regions of *FGFR1, 2, 3* and *TWIST1*. Cytogenetic and array CGH analysis also gave normal result. Our case and the reported ones suggest that this combined phenotype may be related to a variable expressivity of the common mutation of achondroplasia. It can be suspected, that modifier gene(s) may exist in the genome altering the phenotypic outcome of the achondroplasia-specific mutation. In addition, epigenetic or environmental influences may also modify the manifestation of the disease.

6.2 Characteristics of isolated craniosynostoses – predisposing factors

Similarly to previously published studies, nonsyndromic patients represented the majority of all cases with craniosynostosis, and the most frequently involved suture was found to be the sagittal one, followed by the coronal, metopic and lambdoid sutures. The frequency of sagittal synostosis was 68%, higher than in other populations. Assessing the relationship between potential risk factors and craniosynostosis, it was found - in accordance with previous studies - that sagittal synostosis was frequently associated with male sex and twin gestation. Several studies confirmed the role of maternal smoking as a risk factor for craniosynostosis, especially the sagittal type. According to data from the World Health Organization (2011), the estimated prevalence of daily smoking in Hungarian females is

26%, which is much higher than in many other countries (United Kingdom, 14%; United States, 13%; Australia, 15%). Although smoking behavior of mothers was not studied in our patient cohort, we can speculate that smoking can be one of the potential causes of the higher involvement of the sagittal suture in Hungarian patients with craniosynostosis.

6.3. Diagnostic aspects and genetic algorithm

Detailed clinical genetic examination has an important role in not only the recognition the different forms of the disease, but also has a prognostic significance and allows early surgical intervention.

On the basis of our experience and data in the international studies, we established a molecular diagnostic algorithm for the genetic examination of craniosynostosis in the country. If the clinical signs are specific for a certain syndrome, targeted analyses should be performed in a given order to maximize the diagnostic effectivity. If the symptoms propose the syndromic form of craniosynostosis, but they are not specific for a certain syndrome, conventional cytogenetic analysis is recommended at first, followed by the mutational analysis of hotspot regions of the responsible genes.

As the etiology of the isolated group is very heterogeneous and probably is of multifactorial origin, genetic tests are proposed only in certain cases (e.g., involvement of the coronal suture, multiple-suture synostosis, familial cases). From a clinical point of view, it is important, that the knowledge of the genetic background may call our attention to the expected associating clinical signs and anticipate the probable required number of surgical interventions. In genetically determined forms, the probability of refusion, pansynostosis and progression is greater. Reconstruction of the cloverleaf skull in our patients with Apert syndrome recquired 2 or 3 surgical intervetions, while the correction of syndactyly also

needed several operations. Besides the treatment of craniofacial alterations, increased intracranial pressure (hydrocephalus), extreme proptosis and respiratory problems also required surgical interventions in the Pfeiffer syndrome of severe type 2 and 3.

Crouzon, Muenke and Pfeiffer syndrome type 1 are usually characterized by mild cranial deformity. As a result of this, three patients with Muenke syndrome in our cohort did not need any reconstruction.

Additional significance of the genetic diagnosis is the possibility of targeted prenatal testing in familial cases. On the basis of the result, couples can decide the outcome of the pregnancy. As a result of our contribution, three healthy babies have been born so far.

New results of the dissertation

1. We performed comprehensive clinical and genetic investigation of patients with craniosynostosis at first in the country. Taking the advantage of the leader role of the Department of Neurosurgery in craniofacial surgery, we performed a national center for the clinical and genetic examination of patients with craniosynostosis.
2. We achieved expansive genetic examination of syndromic craniosynostoses and established a diagnostic algorithm for effective and economical testing.
3. In 75% of syndromic cases, genetic alterations could be detected.
4. We identified a novel mutation (p.Ser176Arg) in the *TWIST1* gene of a patient with Saethre-Chotzen syndrome, and supported its pathogenecity with additional tests.
5. By the investigation of genotype-phenotype correlations, several observation were made:

- We confirmed the correlation between the type of the mutation and the phenotype in Apert, Crouzon and Pfeiffer syndromes.

- We performed genotype–phenotype analyses in the family of a patient with Pfeiffer syndrome type 1 and p.Pro252Arg mutation and confirmed, that this mutation is associated with variable expressivity.

- We noticed, that specific signs of the syndrome appear only after several generations, which call attention to detailed family studies.

6. We confirmed the previous observation, that the rare association of achondroplasia and multiple-suture craniosynostosis is the consequence of the p.Gly380Arg mutation of *FGFR3*, which supports the role of this mutation both in endochondral and intramembranous ossification.
7. On the basis of statistical analyses, we stated that male sex, twin gestation and very low birth weight are risk factors for non-syndromic craniosynostosis.
8. We observed, that the involvement of sagittal synostosis in isolated patients is significantly higher in the Hungarian population than in other countries, which underlines the significance of further investigation of risk factors.

7. SUMMARY

Craniosynostosis, the premature fusion of cranial sutures, is a clinically and etiologically heterogeneous group of disorders. Both environmental and genetic factors play an important role in the development of isolated (non-syndromic) forms found in the majority of patients, while syndromic cases are frequently caused by gene mutations or more rarely chromosomal aberrations. The aim of our work was to study the clinical and genetic characteristics of patients with craniosynostosis syndromes and to assess the genotype-phenotype correlations. In isolated craniosynostoses, the pathogenetic role of infant sex, birth weight, gestational age and plurality was retrospectively studied.

Using various methods, pathogenic mutations were identified in 75% of syndromic patients. In five cases, the mutations proved to be familial. In the *FGFR1, 2, 3* and *TWIST1* genes, **11 different mutations** were detected. No chromosomal aberrations could be identified. In a patient with Saethre-Chotzen syndrome, a novel pathogenic mutation, **p.Ser176Arg**, in the *TWIST1* gene was found. In a family with Pfeiffer syndrome due to *FGFR1* **p.Pro252Arg** mutation, we observed a variable expressivity with specific signs of the syndrome appearing only in successive generations. In a patient with simultaneous achondroplasia and multiple-suture craniosynostosis, the achondroplasia-specific mutation, **p.Gly380Arg**, was confirmed without any further genetic abnormality suggesting that this mutation has an influence on both endochondral and intramembranous bone formation. Based on our experiments, we developed an algorithm for genetic diagnosis of syndromic craniosynostosis. A relationship between selected perinatal risk factors (**male sex, twin gestation and low birth weight)** and isolated craniosynostosis was found. Clarification of the genetic background of craniosynostosis and establishment of genotype-phenotype correlations are of important diagnostic and prognostic significance offering the possibility for targeted prenatal testing.

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List of publications related to the dissertation

1. **Bessenyei, B.**, Nagy, A., Szakszon, K., Mokánszki, A., Balogh, E., Ujfalusi, A., Tihanyi, M., Novák, L., Bognár, L., Oláh, É.: Clinical and genetic characteristics of craniosynostosis in Hungary. *Am. J. Med. Genet. A. Epub ahead of print (2015)*
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4. Oláh É., **Bessenyei B.**, Kun I., Németh K.: Mendeli öröklődésű kórképek.
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